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Replication Stress: Driver and therapeutic target in genomically instable cancers

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ABSTRACT

Genomically instable cancers are characterized by progressive loss and gain of chromosomal fragments, and the acquisition of complex genomic rearrangements. Such cancers, including triple-negative breast cancers and high-grade serous ovarian cancers, typically show aggressive behavior and lack actionable driver oncogenes. Increasingly, oncogene-induced replication stress or defective replication fork maintenance is considered an important driver of genomic instability. Paradoxically, while replication stress causes chromosomal instability and thereby promotes cancer development, it intrinsically poses a threat to cellular viability. Apparently, tumor cells harboring high levels of replication stress have evolved ways to cope with replication stress. As a consequence, therapeutic targeting of such compensatory mechanisms is likely to preferentially target cancers with high levels of replication stress and may prove useful in potentiating chemotherapeutic approaches that exert their effects by interfering with DNA replication. Here, we discuss ho w replication stress drives chromosomal instability, and the cell cycle-regulated mechanisms that cancer cells employ to deal with replication stress. Importantly, we discuss how mechanisms involving DNA structure-specific resolvases, cell cycle checkpoint kinases and mitotic processing of replication intermediates offer possibilities in developing treatments for difficult-to-treat genomically instable cancers.

1. INTRODUCTION

Recent genomic analyses of triple-negative breast cancers (TNBCs), high-grade serous ovarian cancers (HGSOCs), and other hard-to-treat cancers have underscored the absence of 'druggable' oncogenic drivers.[1–3] Patients with such cancers currently do not benefit from molecularly targeted therapies, and urgently need better treatment options. One characteristic that these tumors share is their profound genomic instability. This phenomenon is characterized by continuous gains and losses of chromosomal fragments and complex genomic rearrangements, usually resulting from defective genome maintenance pathways. As a consequence, genomic instability underlies the rapid acquisition of genomic aberrations that drive therapy failure. Finding novel treatment options for genomically instable cancers is not only relevant for TNBC or HGSOC patients, but also for patients with other hard-to-treat cancers, characterized by extensive genomic instability.

Evidence increasingly points to replication stress as the driver of genomic instability.[4,5] Since replication stress compromises cell viability, cells have apparently evolved various replication stress-resolving mechanisms to mitigate these threats. It is thought that genomically instable tumors increasingly rely on specific mechanisms for their survival, and that these mechanisms could therefore present promising targets for anti-cancer drug development.

Here, we summarize the cancer-associated alterations that lead to replication stress, and discuss the cellular mechanisms that are employed by (tumor) cells to avoid otherwise toxic levels of replication stress. In addition, we will discuss which of these mechanisms could be exploited therapeutically in the treatment of genomically instable cancers.

2. DNA REPLICATION

Licensing and firing

In order to produce a fully duplicated genome, which can be divided over

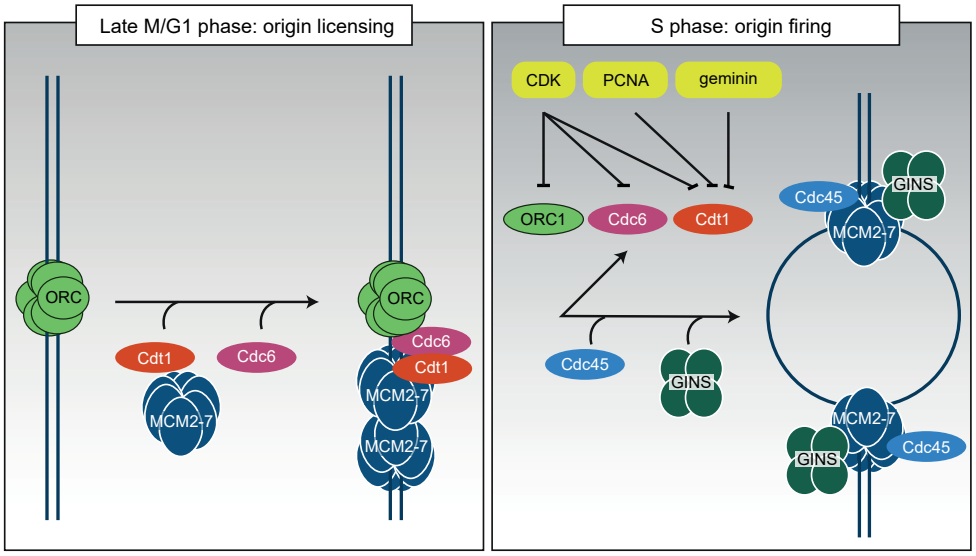

Figure 1. Regulation of origin licensing and firing during replication

Model showing how replication origins are fired only once in the cell cycle. In late mitosis and early G1 phase the pre-replication complex, consisting of the origin recognition complex (orc) with Cdc6, Cdt1 and the inactive helicase MCM2-7 is formed. In S phase, the licensed origins will 'fire' following activation of the MCM2-7 helicase, due to binding of the GINS complex and Cdc45. During origin firing, licensing of origins is inhibited by CDK, PCNA and geminin, which prevents re-replication.

the two daughter cells during mitosis, DNA must be faithfully replicated during S phase. Replication occurs in a bi-directional fashion, and ensues at specific genomic loci, called 'replication origins'. Replication origins are abundantly present in eukaryotic genomes, although their number ranges significantly between species; from ~400 in *S. cerevisiae* to >30,000 in human cells.[6-8] To ensure that the genome is only replicated once per cell cycle, it is vital that initiation of replication at origins is put under strict control. To achieve this, the onset of replication is a two-step process, consisting of 'licensing' and 'firing' of replication origins (Figure 1).

'Licensing' of origins occurs prior to S phase, through the assembly of a pre-replication complex (preRC), consisting of the origin recognition complex (ORC) proteins together with Cdc6, Cdt1 and the inactive helicase hexamer MCM2-7.[9-10] 'Firing' of origins marks the onset of S phase and initiates actual DNA replication. For origin firing, the MCM2-7 helicase is activated by binding of the GINS complex and Cdc45, which together form the CMG complex.[11,12] The activated helicase will initiate DNA unwinding in a bi-directional fashion with 'replication forks' at both ends, creating a so-called 'replication bubble'. Once the DNA helix is unwound, δ polymerase (on the leading strand) and ε polymerase (on the lagging strand) can access the DNA associated to the CMG complex and traverse the DNA strands, allowing for DNA synthesis.[13,14] To maintain genomic integrity, cells need to ensure that 1) the entire genome is only replicated once, and that 2) cells do not proceed cell division before DNA replication is completed. To this end, DNA replication is strictly

controlled by cell cycle regulators, predominantly cyclin-dependent kinases (CDKs). Whereas the abundance of CDKs is relatively constant throughout the cell cycle, their cyclin partners are synthesized and degraded in a cell cycle-dependent manner by the anaphase promoting complex/cyclosome (APC/C). The interplay of CDK and APC/C activity thereby regulates the periodicity of CDK activity.[15,16] In metazoans, over 20 different CDKs have been identified,[17] although only a limited number of CDKs have clear roles in cell cycle regulation and DNA replication.

High CDK activity triggers the firing of origins, which marks the onset of S phase. This is achieved using multiple mechanisms. Firstly, levels of D-type cyclins increase as a consequence of mitogenic signaling, allowing for the activation of CDK4 and CDK6. Once activated, CDK4 and CDK6 deactivate Retinoblastoma (pRB), leading to release of E2F transcription factors, which will transactivate multiple cell cycle regulators, including A and E-type cyclins.[18,19] Subsequent activation of CDKs,[20] as well as the Dbf4-dependent kinase (DDK) drives origin firing.[21-23] Specifically, binding of Cdc45 to the MCM helix complex requires phosphorylation of MCM2-7 by DDK, while simultaneously, CDK-dependent phosphorylation of Treslin is required for proper initiation of DNA replication.[24-26]

In parallel to promoting origin licensing, high CDK activity blocks pre-RC assembly, so that once an origin has fired, it cannot be re-licensed until CDK levels drop during mitotic exit. This mechanism prevents genomic areas from being replicated more than once per cell cycle. Again, this process is achieved in multiple ways. Firstly, the licensing factor Cdt1 is degraded in a manner that requires two distinct E3 ubiquitin ligases. Cdt1 degradation is stimulated by phosphorylation by CDKs and subsequent ubiquitination by SCF-SKP2,[27,28] while binding to PCNA, promotes ubiquitination by Cul4-DDB1.[29,30] In parallel, Cdt1 is inhibited through the binding of geminin.[31-36] Secondly, CDK-mediated phosphorylation of Cdc6 leads to its nuclear exclusion, as well as proteasome-mediated degradation by the SCF.[37,38] Thirdly, the origin-recognition complex component ORC1 is degraded by the SCF-Skp2 when CDK activity is high (Figure 1).[39] Only when CDK activity drops, due to APC/C-mediated degradation of A and B-type cyclins during mitotic exit, a temporal window is created where pre-RC assembly allows for a new round of cell division.[40]

Not all origins are fired simultaneously during S phase. Rather, origin firing adheres to a specific temporal pattern. Major determinants of origin firing are the genomic position of origins and the local chromatin context.[41] Additionally, a number of factors have been identified to regulate origin timing, including Forkhead box transcription factors, as well as RIF1 and TAZ1, originally identified as telomere-binding proteins.[42-44] Presently, only the function of RIF1 in the process of origin timing has been shown to be conserved in mammals.[45-47] While the exact mechanism of origin timing remains elusive, it appears that the spatial organization of the genome plays an important role in genome maintenance.[41] The composition and accessibility of DNA itself also influence replication timing by affecting pre-RC assembly at origins. Indeed, histone composition appears to restrict pre-RC assembly to origins.[48-52]

Whereas clear temporal coordination distinguishes early from late origins, some origins are not fired at all. Under normal conditions, only a subset of the licensed origins is fired, and replication from these origins suffices

to replicate the entire genome. The origins that do not contribute to normal replication are called 'dormant' origins, and genomic regions surrounding dormant origins are passively replicated by forks that initiate from non-dormant origins.[53]

3. REPLICATION STRESS

The term 'replication stress' is defined as the slowing or stalling of replication fork progression.[53] Replication stress can be caused by different factors, many of which are attributed to oncogene activation. Although expression of numerous oncogenes has been linked to the induction of replication stress, we will focus on three oncogenes, which have been studied extensively in the context of replication stress: *MYC*, *CCNE1* and *RAS* (Figure 2).

MYC

One of the oncogenes that was linked early on to replication stress is *MYC*. C-MYC (*MYC*) was originally discovered as the cellular counterpart of the viral V-MYC gene,[54] and belongs to a family of transcription factors, which includes C-MYC, N-MYC, L-MYC and S-MYC in mammals. Of these, C-MYC, N-MYC and L-MYC have been implicated in human tumorigenesis. MYC has transactivating activity, for which it requires interaction with its binding partner MAX.[55] Intriguingly, MYC was later discovered to act both as a transcriptional activator, as well as a transcriptional repressor.[56]

MYC was shown to have oncogenic properties, and overexpression of MYC promotes cell growth,[57] while it blocks cellular differentiation.[58] Moreover, MYC activation alone is sufficient to transform cells, as demonstrated by enhanced MYC expression under immunoglobin enhancers, which induces lymphoma development.[59] In line with these observations, aberrations in the *MYC* gene have been linked to the pathogenesis of a range of cancers, including Burkitt lymphoma,[60,61] diffuse large B-cell lymphoma,[62] as well as breast and prostate cancers.[63]

Induction of proliferation by MYC is thought to be mediated primarily through CDK4/Cyclin D. CDK4 as well as Cyclin D isoforms are direct targets of MYC.[64-66] Conversely, MYC promotes proliferation through repression of cell cycle regulators. Through association with MIZ1, MYC represses CDK inhibitors $p15^{INK4}$ and $p21^{Cip1}$.[67,68] The observation that cells lacking either CDK4 or Cyclin D show a strongly reduced ability to be transformed by MYC further points at CDK4/Cyclin D as an important downstream target in MYC-induced transformation.[69,70] Similarly, Eμ-myc transgenic mice develop lymphomas at slower rates in a CDK2-deficient background.[71] Thus, MYC amplification leads to increased activity of multiple CDKs, which in part underpins MYC-induced proliferation (Figure 2A).

Paradoxically, MYC was recognized to also induce adverse effects on cellular viability. As mentioned above, MYC represses the p53-target $p21^{Cip1}$, which changes the outcome of p53 signaling from cytostatic to pro-apoptotic.[72] Indeed, MYC induction was demonstrated to promote a pro-apoptotic state, and to sensitize cells to death receptor ligands.[73,74]

Importantly, elevation of MYC levels also causes DNA damage, which can be attributed to multiple mechanisms, including the control of DNA replication.[75,76] MYC overexpression was shown to increase transcription of *CDT1*, which is crucially required for the loading of the MCM complex to replication origins.[6,9] Notably, CDT1 overexpression can induce cellular transformation,

suggesting that upregulation of CDT1 by MYC plays a role in MYC-mediated tumorigenesis.[76] In addition to the transcriptional control of DNA replication, MYC has been described to fuel the initiation of DNA replication through a non-transcriptional manner. Specifically, MYC interacts with MCM proteins, including MCM2 and MCM7, which leads to increased replication origin activity, and replication-dependent DNA damage (Figure 2A).[77] Of note, MYC was shown to promote efficient replication in cell-free Xenopus extracts, devoid of RNA transcription, underscoring the non-transcriptional role of MYC in this process.[77] In line with these findings, the non-transcriptional effects of MYC on replication were shown to require CDC45.[75] Combined, MYC overexpression is responsible for unscheduled origin firing – both through transcriptional as well as non-transcriptional ways- and leads to replication-dependent DNA lesions.

An alternative way through which MYC overexpression adversely impacts cellular viability is the elevation of reactive oxygen species (ROS) (Figure 2B).[78] Importantly, the amount of MYC-induced DNA lesions correlated with ROS levels, and treatment with the anti-oxidant NAC lowered ROS levels and prevented the formation of DNA lesions.[78] Simultaneously, MYC overexpression disrupts the proper resolution of DNA lesions, including DNA double strand breaks (DSBs) by interfering with DNA repair.[79] Unclear, however, is which specific DNA repair pathway is affected by MYC.[79] Taken together, these mechanisms explain the observed DDR activation, genomic instability and cellular senescence upon MYC overexpression.[71,80,81]

Cyclin E

Another oncogene that has been connected to induction of replication stress is Cyclin E, the gene-product of *CCNE1*. Cyclin E contributes to the transition from G1 to S phase by binding and elevating the activity of CDK2.[82-84] Consequently, CDK2 phosphorylates Retinoblastoma (RB) to release E2F transcription factors, which stimulate S phase entry by transactivating multiple genes required for DNA replication.[85,86] Importantly, Cyclin E expression is under control by other pro-oncogenes, including MYC,[87] so Cyclin E-mediated effects can be indirect consequences of other oncogenic events.

High levels of Cyclin E–CDK2 were shown to profoundly influence replication dynamics. Indeed, Cyclin E overexpression impairs the loading of MCM proteins including MCM2, MCM4 and MCM7.[88] As a consequence, Cyclin E overexpression causes inefficient pre-replication complex formation and negatively impacts replication initiation, as judged by BrdU incorporation and PCNA foci formation.[88] Furthermore, elevated levels of CDK2 activity that accompany Cyclin E overexpression increases the rate of origin firing (Figure 2A). These increased rates of origin firing consequently lead to depletion of the nucleotide pool,[89] in parallel to inducing collisions between the replication machinery and the transcription complexes.[90] These combined mechanisms underlie the perturbed replication dynamics upon Cyclin E overexpression, and explain the observed replication-dependent DNA lesions and activation of the DNA damage response (DDR).[5,91] Thus, overexpression of Cyclin E, in analogy to c-MYC overexpression, was shown to accelerate S phase entry, while it counter-intuitively results in a reduced rate of DNA synthesis.[92,93] In line with the notion that replication failure can induce structural and numerical chromosome abnormalities,[94] karyotypic analysis showed that Cyclin E deregulation affects the fidelity of chromosome transmission, resulting in

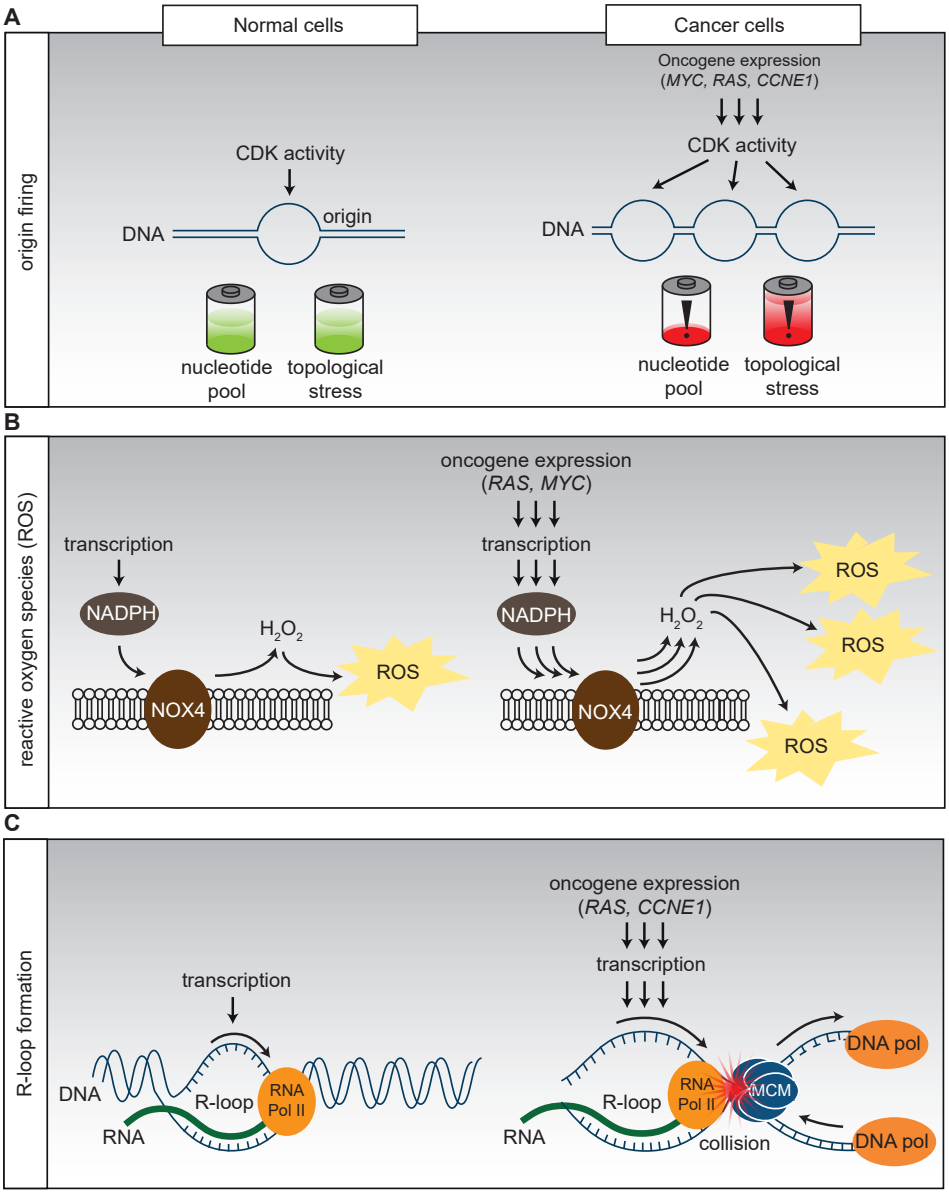

Figure 2. Sources of replication stress in cancer cells

Various cellular mechanisms underlie replication stress in cancer cells. Key examples are shown. ***A****) In normal cells, initiation of DNA replication adheres to a specific and coordinated temporal program. In cancer cells, oncogene expression leads to unscheduled origin firing, and consequent nucleotide pool exhaustion. In addition, excessive origin firing increases DNA topological stress.* ***B****) Reactive oxygen species (ROS) are natural by-products of cellular metabolism, and mediate signal transduction. Oncogene activation in cancer cells can lead to aberrant transcription of proteins involved in cellular metabolism, resulting in increased production of ROS and ensuing replication stress.* ***C****) R-loops are formed when nascent RNA from transcription interacts with DNA. Increased transcriptional activity in cancer cells leads to elevated levels of R-loops, which can collide with the DNA replication machinery at replication forks.*

genomic instability.[95]

RAS

The RAS family of GTPases comprises three genes: *H-RAS*, *K-RAS* and *N-RAS*.[96] RAS acts a pivotal signal transducer between receptor-tyrosine-kinases (RTKs) and the mitogen-activated protein kinase (MAPK) cascade, which culminates in the activation of a complex transcriptional program, including the activation of c-Jun/c-Fos transcription factors. One of the transcriptional targets of c-Jun/c-Fos is Cyclin D,[97] which underpins cell cycle entry in response to RAS signaling.[98]

RAS isoforms were shown to be mutated in multiple cancer subtypes, and involve common point-mutations that turn RAS into an active oncogene.[96] Expression of one such RAS mutant, H-RAS-V12 was shown to induce replication stress. Specifically, expression of H-RAS-V12 induces the number of active replication origins, leading to DDR activation and triggering senescence in non-transformed cells.[99] The mechanisms through which overexpression of oncogenic RAS induces replication stress are only partly understood. In line with RAS inducing MAPK signaling, various studies have revealed that oncogenic RAS is responsible for accelerated cell growth and increasing the fraction of cells in S phase.[100,101] Simultaneously, and again in line with above-mentioned oncogenes, oncogenic RAS elevates transcriptional activity and leads to collisions of transcriptional components with the replication machinery, which causes replication stress (Figure 2C).[102] Another consequence of oncogenic RAS signaling, that compromises DNA replication, is increased ROS production (Figure 2B).[101,103] Specifically, oncogenic RAS elevates the mRNA level of the NADPH oxidase NOX4, which in turn leads to increased H2O2 generation.[104] RAS-mediated ROS leads to damaged DNA, as evidenced by increased levels of 8-oxoguanine.[101] Increased oxidative damage to RNA/DNA was demonstrated to interfere with replication fork velocity in response to oncogenic RAS,[105] in line with elevated levels of γ-H2AX and 53BP1,[89,101] and chromosomal instability.[106]

Based on findings on MYC, Cyclin E and RAS oncogenes, multiple common themes appear to underlie oncogene-induced replication stress. One of these common mechanisms is depletion of the nucleotide pool (Figure 2A). When cells do not adhere to the temporal and spatial program of origin firing due to elevated CDK2 activity, both early and late origins are fired simultaneously. Additionally, dormant origins may be fired in an unscheduled manner.[90] More recently, the Halazonetis lab showed that Cyclin E or MYC overexpression leads to *de novo* origin replication sites, preferentially located in highly transcribed genes.[107] The increased levels of replication subsequently result in nucleotide pool exhaustion.[89] Consequently, insufficient pools of nucleotides induce replication stress and can subsequently cause chromosomal instability.[90] In line with limited nucleotide supply hampering replication fidelity, oncogene-induced DNA damage was shown to be rescued by supplying exogenous nucleosides.[89]

An additional common source of replication stress is the increased level of DNA-RNA hybrids, called R-loops (Figure 2C).[108] R-loops form when nascently transcribed mRNA anneals to its complementary DNA strand.[109] The resulting three-stranded structure consists of a DNA-RNA hybrid, and a displaced single DNA strand.[110] R-Loops have been shown to result from RNA polymerase-II (RNA POL-II)-mediated transcription,[111] but can also occur at highly active RNA POL-I-transcribed regions of rDNA.[112] The formation of R-loops is influenced by

G-rich RNA, the extent of supercoiling and the presence of nicks in DNA.[113] Since the discovery of DNA-RNA hybrids, multiple studies have confirmed the implication of R-loops in biological processes such as mitochondrial DNA replication,[114-116] and transcription.[117] Importantly, R-loops can become an endogenous source of replication stress, if they pose a barrier to fork progression.[102,118-120] In line with many oncogenes inducing transcription, oncogene overexpression or oncogenic mutations were shown to correlate with increased DNA-RNA collisions. Specifically, the RNA synthesis stimulated upon overexpression of Cyclin E or HRAS mutation was shown to result in R-loop accumulation and ensuing DNA damage (Figure 2C).[102]

Increased transcriptional activity and the ensuing R-loop formation may lead to collisions of DNA-RNA hybrids with the replication machinery.[53] Especially at long genes, R-loops can interfere with replication and lead to the expression of common fragile sites (CFS).[121] Of note, R-loop accumulation is found to be orientation-dependent, with replisomes oriented head-on with RNA polymerases creating R-loops, in contrast to co-directional replisomes.[119] As discussed above, enhanced oncogene expression was shown to induce firing of ectopic origins, mainly located in highly transcribed genes.[107] In this situation, a local increase in both replication forks as well as R-loops underlies replication fork collapse and DNA double-strand break formation.[107]

Accumulation of R-loops and ensuing replication stress is not solely linked to specific oncogenes, but also arises in response to mitogen-induced signaling. For instance, estrogen-dependent transcription was shown to underpin R-loop-mediated replication stress and genomic instability in estrogen-driven breast cancers.[122]

Combined, multiple oncogenes were shown to induce replication stress, which involves common mechanisms, including nucleotide pool depletion and R-loop formation.

4. HOW TO DEAL WITH REPLICATION STRESS

ATR-CHK1 signaling

In order to deal with replication stress, cells have evolved mechanisms to monitor and respond to stalled replication, often referred to as the replication checkpoint or intra-S phase checkpoint (Figure 3). Slowing or stalling of replication forks typically results in long stretches of ssDNA, which are rapidly coated by the Replication Protein A (RPA) protein trimer.[123] Subsequently, RPA enables the recruitment of ATR, the central orchestrator of the replication stress response. Indeed, ATR activation was shown to require replication forks,[124] and the formation of excessive amounts of single-stranded DNA.[125,126] ssDNA at stalled replication forks arises because the DNA helicase and DNA polymerase are uncoupled (Figure 3).[127] The ensuing RPA-coated ssDNA tracks are then recognized by the ATR interactor ATRIP, leading to the recruitment of ATR-ATRIP to chromatin.[126] ATR and ATRIP are dependent on each other for their stability. Therefore, ATRIP loss phenocopies ATR inactivation, and results in sensitivity to DNA damage, loss of ATR phosphorylation and loss of cellular viability.[128]

However, localization of the ATRIP-ATR complex to RPA-coated ssDNA is not sufficient for ATR activation. Two parallel pathways exist that initiate ATR activation. Firstly, ATR is activated by the ring-shaped 9-1-1 protein complex, consisting of RAD9, RAD1 and HUS1 (also called the CLAMP complex). Mechanistically, the 9-1-1 complex

recognizes the 5'-end of ssDNA, adjacent to RPA, and is subsequently loaded onto DNA by the Rad17/Rfc2-5 replication factor complex.[129-130] Through this mechanism, ATR is activated specifically at ssDNA-dsDNA junctions, which characterize stalled replication forks during replication stress. In a subsequent step, the 9-1-1 complex facilitates ATR activation by recruitment of Topoisomerase-binding protein-1 (TOPBP1). Secondly, using a parallel mechanism, ETAA1 activates ATR independently of TOPBP1. ETAA1 directly interacts with RPA, at both unperturbed and stalled replication forks.[131-132] Once ATR is activated, it phosphorylates a plethora of downstream targets, initiating various responses to maintain genome integrity (Figure 3).[133] An important initial response to replication stress is to halt cell cycle progression, allowing time to resolve lesions or to complete replication. The cell cycle checkpoint arrest following replication stress is, in large part, dependent on the activation of the ATR substrate CHK1. CHK1 activation requires Claspin, which brings CHK1 in close proximity to ATR.[134] In turn, phosphorylated CHK1 will activate WEE1,[135] while it inactivates the CDC25A, CDC25B and CDC25C phosphatases.[136-139] Through these combined effects, ATR/CHK1 signaling prevents the activation of CDK1 and CDK2, resulting in a S phase and G2 phase cell cycle checkpoint arrest (Figure 3). Furthermore, ATR activation leads to stabilization of p53, which induces a transcriptional program, triggering upregulation of the CDK inhibitor p21.[140,141] Combined, ATR signaling leads to the loss of CDK-activation, while CDK inhibitory proteins are upregulated, leading to arrested cell cycle progression.

Although studied less intensively, a parallel mechanism for cell cycle checkpoint inactivation involves MAP kinase-activated protein kinase-2 (MK-2). MK-2 is required to install a DNA damage-induced cell cycle arrest, especially in the context of defective p53 signaling.[142,143] Additionally, MK-2 was found to be responsible for lowering replication dynamics in situations of replication stress.[144]

Beyond induction of a cell cycle arrest, a major downstream consequence of ATR and CHK1 activation involves the regulation of replication origin firing. In response to ssDNA accumulation, both ATR, CHK1 and WEE1 limit the firing of replication origins, mainly during early S phase (Figure 3).[145] As a consequence, inactivation of ATR or CHK1 in cells leads to increased origin firing, both in the absence or presence of replication blocking agents.[145-148] Mechanistically, ATR/CHK1 signaling locally prevents replication origin firing following replication stress by interfering with the binding of CDC45 to the MCM2-7 helicase.[149-150] Conversely, CHK1 appears to be involved in the activation of dormant origins.[151] These effects could be mediated through modification of MCM helicase components present at dormant origins. Additionally, phosphorylation of FANC-I by ATR was shown to actually prevent dormant origin firing, underscoring the complex regulation of this process.[152] The global inhibition of replication initiation at new replication factories by ATR/CHK1 signaling thus directs replication away from regions that have yet to start replication, and towards initiation of dormant factories at regions where forks are stalled.[153]

Signaling through ATR and CHK1 further contributes to preventing genomic instability, by stabilizing stalled replication forks. Specifically, ATR/CHK1 prevent the nuclease-dependent regression of stalled replication forks.[154,155] Exactly how ATR facilitates fork stability is not completely clear, but the

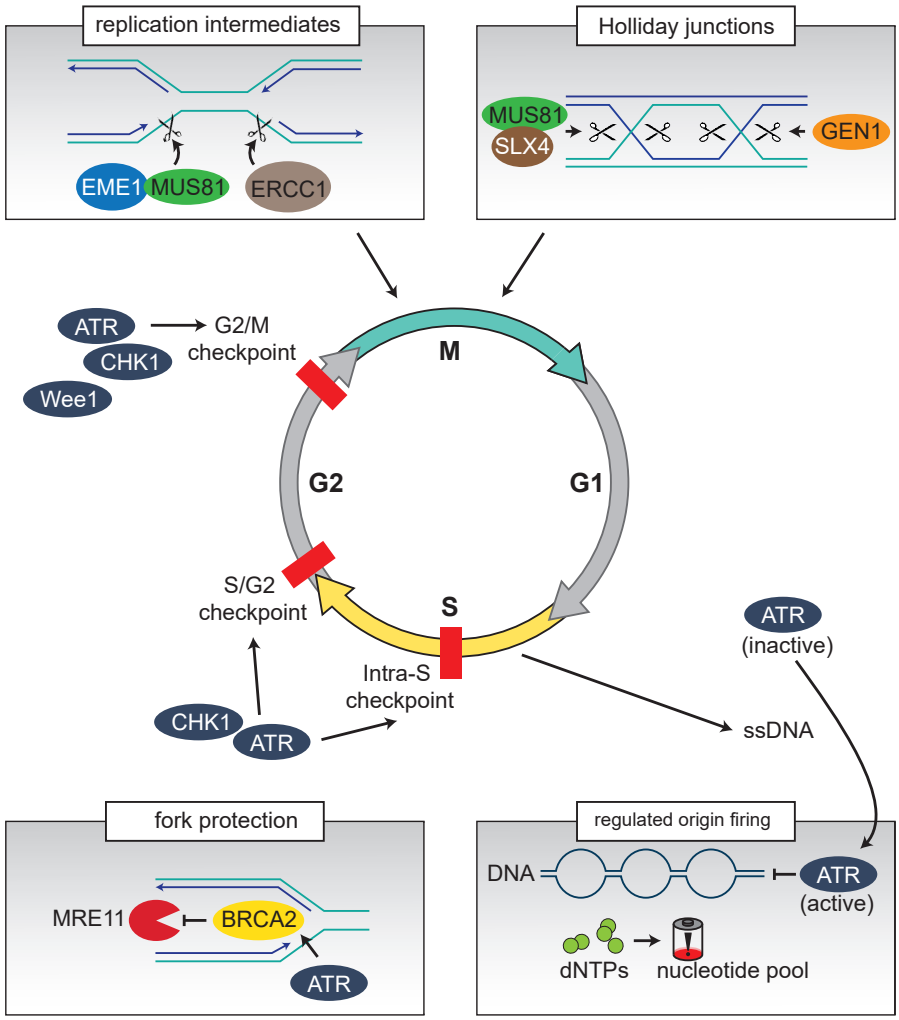

Figure 3. Mechanisms to deal with replication stress

A distinct feature of replication stress is the excessive levels of single-stranded DNA (ssDNA). ssDNA is coated by RPA, which in turn recruits multiple proteins and leads to activation of ATR and its substrate CHK1. ATR/CHK1 signaling can halt the cell cycle at different phases, as indicated. Through ssDNA accumulation, ATR limits the firing of replication origins to prevent further fork stalling and nucleotide pool depletion. In addition, cells in which replication forks stall must protect their nascent DNA from MRE11-mediated exonuclease activity. To do so, ATR coordinates homology-directed repair, in which RAD51, BRCA1 and BRCA2 are key components of fork protection. To maintain genome stability, cells can process late replication intermediates in mitosis through the action of EME1-MUS81 and ERCC1. In addition, unresolved mitotic Holliday junctions can also be resolved in mitosis by SLX4-MUS81 or GEN1 endonuclease activity.

regulation of SMARCAL1 and binding of FANCD2 to the MCM2-7 complex at forks are thought to be important.[156,157] Indeed, inhibition of ATR was found to increase fork regression by inhibiting SMARCAL1 mediated fork reversal.[156] Additionally, CHK1 prevents MUS81-mediated fork collapse.[158-160] In fact, it was reported that ssDNA stretches at stalled replication forks can hybridize and result in a four-way structure termed 'reversed fork' or 'chicken-foot like structure'.[161,162] The formation of reversed forks halts replication, thereby preventing deleterious fork progression during stressed conditions, allowing for time to deal with such lesions.[163]

In human cells, fork reversal occurs following different genotoxic agents, and therefore likely represents a generic response to replication stress.[163] Fork reversal is catalyzed by numerous DNA translocases and helicases, including SMARCAL1, ZRANB3, HLTF, BLM, FANC-M, FANC-J and WRN.[163] Furthermore, the process of fork reversal is regulated by PARP.[164] Specifically, inhibition of PARP resulted in an increase of RECQ1-mediated fork restart and thus less reversed forks.[163] Fork reversal therefore, seems to be a carefully regulated process in cells to transiently stall replication forks during replication stress. Possibly, fork reversal provides a mechanism to prevent permanent stalling of forks, if they cannot be properly restarted.[53,163]

Replication fork protection

Once replication forks are stalled, the nascent DNA at forks must be protected from nucleolytic cleavage and nuclease-mediated degradation. Indeed, recent data suggest that reversed forks are acted upon by a range of nucleases, including MRE11, SLX4 and MUS81, resulting in fork collapse and DNA breaks. The above-mentioned nucleases only degrade stalled replication forks when replication fork protection is defective. Currently, two separate fork protection pathways have been identified. The first entails the protection of nascent DNA by BRCA1, BRCA2 and FANCD2 against degradation by MRE11 (Figure 3).[165,166] Mechanistically, the role of BRCA2 and FANCD2 in replication fork protection is speculated to involve recruitment to and stabilization of RAD51 at stalled forks.[166-168] Yet, RAD51 was more recently shown to also be required for the reversal of stalled forks, a key intermediate step in fork degradation underscoring a dual role of RAD51.[169] The recruitment of the endo/exonuclease MRE11 to stalled forks was further shown to depend on PARP1,[170] as well as PTIP, MLL3/4 and Cdh4,[171] and leads to the degradation of nascent DNA at unprotected, reversed replications forks.[169] BRCA2 and FANCD2 also protect stalled forks from degradation of nascent DNA by the MUS81 nuclease, independently of MRE11.[172] Mechanistically, MUS81 recruitment to stalled forks requires methylation of lysine 27 on Histone H3, and the polycomb components EZH2.[172]

A second protection pathway involves the protein ABRO1, which protects DNA at stalled forks from degradation by the DNA2 nuclease and the WRN helicase.[173] Notably, this pathway operates independently of RAD51 filament stabilization. Rather, inactivation of RAD51 rescued DNA2-mediated fork degradation in cells lacking ABRO1.[173] This latter observation is likely reflecting the role of RAD51 in promoting fork reversal, in line with the selective targeting of reversed forks by DNA2.[173,174]

How exactly replication forks are protected, and what the molecular steps are in fork degradation remains elusive. Additionally, it is still unclear to what extent protection of stalled replication forks is required for viability of normal cells, since the HR-related

function rather than the fork protection function of BRCA2 was shown to underpin the lethality upon BRCA2 loss.[175] Nevertheless, replication fork protection appears to become important when HR-deficient cancer cells are treated with replication-blocking agents, since mutations that rescue fork protection lead to treatment resistance.[171,172]

Homologous recombination repair

If stalled replication forks break, they produce single-ended, double-stranded DNA breaks (DSBs), which can be extremely toxic if left unrepaired. In order to repair these lesions and preserve genomic stability, the homologous recombination (HR) machinery is crucial.[176] HR repair utilizes a homologous DNA template, usually the sister chromatid, allowing for relatively error-free repair.[177] For HR to occur, initial processing of DSBs is required, wherein the 5' terminus of a DNA strand break is resected to generate 3' ssDNA overhangs. To achieve this, the endonuclease activity of the MRE11/RAD50/NBS1 (MRN) complex in conjunction with CtIP/BRCA1 makes an initial cut close to the break sit and performs end-resection towards the break.[178,179] Subsequently, the EXO1 and DNA2 exonucleases perform extensive end-resection to yield long stretches of ssDNA.[179] In a BRCA2-dependent process, RAD51 filaments are formed onto ssDNA, which perform the homology search and recombination.[171,180] The resulting joint DNA molecules, termed Holliday junctions (HJs), require timely resolution to enable proper chromosome segregation (Figure 3). HJs formed by recombinational repair in mitotic cells are preferentially processed by topoisomerase-mediated dissolution by the BTR complex, consisting of BLM-TopoIIIα-RMI1-RMI2,[68,181,182] leading to non-cross-overs. Alternatively, HJs can be resolved, through resolution pathways, involving the endonucleases SLX1-SLX4 and MUS81-EME1.[183]

The Fanconi anemia pathway, translesion synthesis and alternative end-joining

Besides homologous recombination repair, multiple additional repair pathways are involved in the resolution of replication-blocking lesions. In response to crosslinking DNA lesions, the Fanconi anemia (FA) pathway is activated. Fanconi anemia consists of >20 genes, with new Fanconi anemia genes still being identified.[184] Of note, various FA genes also function in other DNA repair pathways, including the HR genes BRCA1 (FANCS), BRCA2 (FANCD1) and PALB2 (FANCN).[185-187] Mechanistically, the majority of the FA proteins assemble to form the FA core complex, which functions as an E3 ubiquitin ligase.[188] The substrate of the FA core complex is the FANCI/FANCD2 complex, that upon ubiquitylation associates with chromatin in DNA repair foci, to repair DNA lesions in concert with downstream FA components and additional DNA repair pathways.

In keeping with a role for FA proteins to resolve replication blocking DNA lesions, cancer cells with FA defects are known to be exquisitely sensitive to crosslinking agents such as cisplatin and mitomycin C,[189,190] but also to PARP1 inhibitors, all known to interfere with replication fork dynamics.[191] Conversely, cancer cells with high levels of replication stress likely depend increasingly on FA components for their survival, since FA components are required for the cellular response to replication stress, including replication fork protection and processing of late-stage replication intermediates during mitosis.[166,186,192]

Translesion synthesis (TLS) also allows cells to deal with increased levels of replication stress.[193] TLS

involves replacement of 'regular' DNA polymerases with specific DNA polymerases, with larger active sites that allow incorporation of bases opposite to damaged nucleotides. A key factor that facilitates polymerase switching is the *proliferating cell nuclear antigen* (PCNA).[194] Upon encountering a DNA lesion, PCNA is mono-ubiquitylated by RAD18/RAD6.[195,196] Subsequently, TLS polymerases bind ubiquitylated PCNA, which results in their recruitment to sites of damaged DNA during replication.[196,197] Rather than a DNA repair pathway, TLS is a DNA damage tolerance (DDT) pathway that tumors may depend on for their survival.[198] While TLS allows cells to proliferate with otherwise replication-blocking DNA lesions, it simultaneously facilitates mutagenesis since TLS polymerases typically have lower fidelity when compared to 'regular' polymerases.

A specific translesion polymerase is polymerase theta (Pol theta), encoded by the POLQ gene. Beyond its role in TLS, Pol theta is required for alternate end-joining (AltEJ) of DNA double strand breaks.[199] Pol theta can ligate resected DNA ends, only requires micro-homology and thereby functions as an alternative repair option to HR repair. In comparison to HR, Pol theta-mediated repair causes genomic rearrangements, leading distinct genomic signatures. Notably, POLQ has been described to be upregulated in multiple tumor subtypes.[200] More recently, inactivation of Pol theta was found to be synthetic lethal with HR mutations,[201] and targeting of Pol theta may therefore be an attractive therapeutic avenue for HR-deficient cancers. Intriguingly, inactivation of Pol theta in HR-proficient cancer cells was reported to result in enhanced sensitivity to replication stress-inducing agents, indicating that Pol theta might have a role in allowing cancer cells to deal with high levels of replication stress.[202]

Mitotic processing of replication-born lesions

Despite the above-mentioned mechanisms that enable cells to deal with RS, replication lesions frequently are left unrepaired and are transmitted into mitosis.[203,204] Such persisting DNA lesions need to be resolved in order to allow sister chromatids to be properly distributed over daughter cells. To do so, cells have developed pathways that can resolve these lesions during mitosis. Resolution of remaining joint molecules in mitosis is conducted by MUS81, GEN1 and SLX4 (Figure 3).[183] The processive activity of these nucleases is upregulated by two distinct mechanisms. Firstly, a holoenzyme is formed by the association of SLX1-SLX4 and MUS81-EME1 with the scaffold protein SLX1. The activity of this holoenzyme is stimulated by the mitotic kinases CDK1 and polo-like kinase-1 (PLK1).[205] In fact, the SLX1 scaffold recruits several additional DNA processing enzymes, including XPF-ERCC1, MSH2-MSH3, TRF2-RAP1 and SNM1B/Apollo, to form a mitotic endo/exonuclease able to resolve a variety of DNA lesions.[205] Secondly, HJs that remain unresolved prior to mitotic entry can be processed by the canonical HJ resolvase GEN1 (Figure 3). During interphase, GEN1 is excluded from the nucleus through a strong nuclear exclusion signal. Upon nuclear envelope breakdown during mitotic onset, GEN1 gains access to mitotic chromosomes, allowing joint molecules resolution.[206]

In situations of replication stress, distinct genomic regions (referred to as common fragile sites, CFSs) may remain under-replicated. Upon mitotic entry, these late-stage replication intermediates are processed by MUS81-EME1 and ERCC1 (Figure 3).[207,208] Specifically, the MUS81 endonuclease is recruited to CFSs in mitosis, allowing

for POLD3-dependent replication at these sites, thereby preventing severe genomic instability.[203] To prevent these mitotic nuclease activities from damaging DNA during S phase, the targeting of MUS81 to lesions seems dependent on binding to SLX4 after phosphorylation of SLX4 by CDK1.[205] Indeed, when CDK is activated prematurely through WEE1 inhibition, complex formation between MUS81 and SLX4 is stimulated, resulting in pulverized chromosomes and cell death.[209]

When joint molecules remain unresolved at anaphase onset, they become visible as ultra-fine bridges (UFBs).[192] These structures arise due to multiple problems, including catenated DNA at centromeric regions, under-replicated regions at chromosome arms, and unresolved HJs.[210-212] When UFBs arise, the PICH DNA translocase binds these DNA regions under tension, and subsequently recruits the BTRR complex,[213-215] as well as RIF1.[216] Replication stress-induced UFBs undergo BLM-dependent processing to create ssDNA at UFBs, as judged by the recruitment of RPA.[210,216] It is speculated that the generation of ssDNA - which is less rigid than dsDNA - enables UFBs to be broken and allows for the seperation of daughter cells during cytokinesis, albeit at the cost of generating DNA lesions.[210] The impact of UFB processing mechanism on genome stability becomes strikingly evident in cells lacking their critical components. Indeed, cells lacking either PICH, RIF1 or BLM accumulate micronuclei,[216] which are known to frequently lead to genomic rearrangements.[217]

Taken together, cells have evolved several sophisticated mechanisms to resolve potentially toxic genomic lesions that are transmitted into mitosis, and to safeguard mitotic progression and genomic integrity.

5. TARGETING REPLICATION STRESS IN CANCER

The replication stress that was observed upon expression of oncogenes *in vitro* also appears to be a highly relevant phenomenon in cancer development. Expression of oncogenes, including Cyclin E, in early neoplastic lesions was shown to coincide with activation of DNA damage response (DDR) markers and arrested proliferation.[91,218] In malignant lesions, the DNA damage response was no longer activated, likely due to p53 inactivation. Combined, these results suggested that the induction of replication stress by oncogene activation in early oncogenesis leads to a DNA damage response and ensuing cell cycle arrest.[5,91] These results also explain earlier observations in which expression of oncogenes, including RAS-V12 and c-MYC in mouse embryonic fibroblasts (MEFs), induced a block in proliferation,[219-221] which was rescued by p53 inactivation.[221]

The loss of p53 signaling is common in cancer, and leads to loss of G1/S cell cycle checkpoint control.[222] As a consequence, *TP53* mutant cancer cells increasingly depend on their G2/M checkpoint to sustain viability in situations of DNA damage. Especially in situations of oncogene-induced replication stress, with concomitant loss of p53, tumor cells likely have an increased dependence on remaining cell cycle checkpoint components, as well as the above-mentioned pathways that resolve DNA replication lesions. Therefore, these pathways are potential therapeutic targets in cancer treatment, especially for those cancers that suffer highly from replication stress. Below, therapeutic strategies are discussed which could be exploited to target cancer cells with high levels of replication stress.

Induction of replication catastrophe

Perhaps the most straightforward possibility for cancer cell eradication is to either therapeutically enhance replication stress using certain agents or by inhibiting the replication stress response checkpoint (Figure 4). In cancer cells with high intrinsic replication stress, this will result in replication stress overload, already during S phase,[223] inducing cell death termed 'replication catastrophe'.[224,225] Mechanistically, replication catastrophe ensues when insufficient RPA is available to coat and thereby protect the high amounts of ssDNA arising as a consequence of fork stalling.[224] Subsequently, the unprotected ssDNA will result in DSB formation and cell death. Interestingly, RPA exhaustion, and the resulting replication catastrophe, can be induced by prolonged treatment with different replication stress-inducing agents, including HU, gemcitabine, cytarabine, aphidicolin, UV light and methyl methanesulfonate,[225,226] and could be exacerbated by combined treatment with inhibitors of the ATR, WEE1 or CHK1 checkpoint kinases (Figure 4).[225]

Targeting oxidized nucleosides

As mentioned above, cancer cells suffer from depletion of nucleotide levels due to oncogene-mediated increased origin firing. In addition, the available nucleotides can be oxidized due to elevated levels of ROS.[227] Incorporation of oxidized nucleotides into the genome has been associated with the generation of DNA mismatches, mutations, and can lead to cell death.[228,229] The MTH1 protein processes oxidized nucleotides, and thereby sanitizes the nucleotide pool, preventing DNA damage.[230] Interestingly, transformed cells often overexpress MTH1 to cope with elevated levels of oxidized deoxynucleoside-triphosphates (dNTPs).[231] More importantly, inhibition of MTH1 was found to be essential for survival of cancer cells.[231] Possibly, cancer cells with high levels of replication stress through oxidized nucleotides -for instance due to MYC amplification- could be targeted by MTH1 inhibition.

Targeting cell cycle checkpoint kinases

If cells with high levels of replicative lesions do not initiate cell death, they will likely rely on checkpoint-mediated G2-M cell cycle delay to provide ample time to deal with such lesions.[232] Abrogation of a G2-M cell cycle arrest could therefore cause cancer cells to enter mitosis prematurely, resulting in mitotic aberrancies and cell death.[233-235] In the context of replication stress, the main cell cycle checkpoint mediators for inducing G2-M delay or arrest are ATR, CHK1 and WEE1, and could therefore be promising targets to target cancers with high levels of replication stress (Figure 4). Initial experiments that showed that cell cycle checkpoint inhibition sensitized cancer cells to DNA damaging agents employed caffeine, a non-selective inhibitor of the ATR and ATM kinases. In the last decade, multiple potent and selective inhibitors to cell cycle checkpoint kinases have been developed, and are currently being tested in preclinical and clinical settings (reviewed elsewhere).[236]

Targeting cell cycle checkpoint kinases indeed appears to be a powerful approach for cancers with high levels of replication stress. Tumors with oncogene-induced replication stress, as induced by H-RAS^{G12V} and K-RAS^{G12D} mutations or overexpression of C-MYC, failed to grow following hypomorphic suppression of ATR.[237] Additionally, ATR and CHK1 inhibitors selectively killed MYC-driven tumors.[238] These findings can be explained, at least in part, by a role of ATR and CHK1 in facilitating a checkpoint arrest. In line with this notion, resistance to ATR

inhibitors was observed following genetic inactivation of CDC25A, in which case cells no longer enter mitosis prematurely.[239] Yet, also DNA damage accumulation in response to CHK1 inhibition is reversed by CDC25A inactivation, suggesting that targeting these checkpoint kinases works through combined induction of DNA damage and G2/M checkpoint override. Similar findings were observed for Cyclin E-overexpressing tumors treated with WEE1 inhibitors,[240] and K-RAS-mutant tumors, which showed profound mitotic catastrophe in response to combined treatment with CHK1 and MK2 inhibitors.[241] Interestingly, ATR was also found to have a role in mitosis, independent of its interphase functions.[242] By acting upon RPA-coated R-loops, ATR was found to prevent lagging chromosome formation at centrosomes, thereby ensuring faithful chromosome segregation.[242]

Clearly, ATR-CHK1, and also WEE1, have essential functions in multiple mechanisms utilized by cancer cells to deal with replication stress. In line with this notion, checkpoint kinases, including ATR and WEE1, were found to be upregulated in numerous cancers.[235,243,244] Likely, the mechanisms

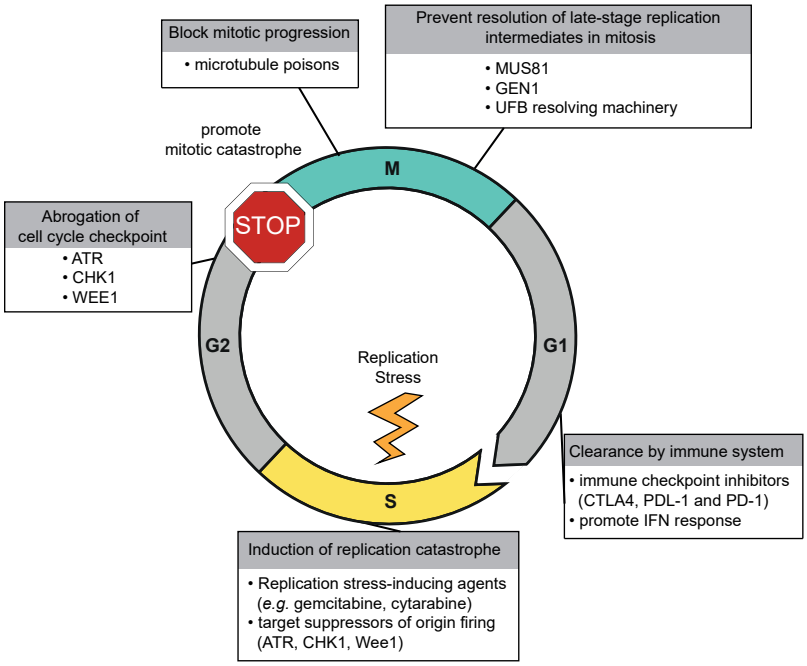

Figure 4. Targeting replication stress in cancer

Cancer cells harboring replication stress could be targeted at different stages of the cell cycle, through numerous mechanisms. Firstly, replication stress could be enhanced in cancer cells that already suffer from high levels of replication stress to induce replication catastrophe. Secondly, cancer cells could be targeted by abrogating their G2-M cell cycle checkpoint. Through this approach, cancer cells with replication-born lesions prematurely enter mitosis, inducing mitotic catastrophe. Additionally, cancer cells in which replication-mediated DNA lesions have been propagated into mitosis might depend on the activity of resolvases, targeting of which might further promote mitotic catastrophe. Thirdly, replication stress leads to mitotic aberrancies and subsequent formation of micronuclei. Upon rupture, micronuclei release DNA into the cytoplasm and triggers cGAS/STING-dependent interferon signaling. Interferon signaling may subsequently prime tumors for immune checkpoint inhibitors.

that underlie the cytotoxic effects of targeting ATR, CHK1 and WEE1 involve multiple interdependent effects, possibly explaining their success in eradicating cells with replication stress.

Targeting replication stress in mitosis

Interestingly, however, cells with replication stress regularly do not arrest at the G2-M checkpoint. Indeed, it is becoming increasingly clear that replication stress is often unresolved prior to mitotic entry, resulting in aberrancies including chromatin bridges and lagging chromosomes.[192,204,207,210] The targeting of resolvase pathways is therefore likely to aggravate replication stress-induced mitotic aberrancies in cancer cells, ultimately resulting in a failure to complete cytokinesis, inducing multinucleation and cell death (Figure 4). A recent study underscored the dependence on resolvases in situations of late-stage replication intermediates in mitosis. Specifically, BRCA2-deficient cells were found to enter mitosis with under-replicated regions, resulting in chromatin bridges in anaphase.[245] Depletion of MUS81 in BRCA2-defective cells further enhanced this mitotic phenotype, and induced multinucleation and cell death.[245] Furthermore, loss of resolvase activity due to combined inactivation of MUS81 and GEN1 leads to ultra-fine bridges, chromosomal rearrangements and cell death (Figure 4).[210] Of note, exacerbating mitotic aberrancies in BRCA2-defective cells was observed in response to PARP inhibition, and the aberrant mitoses upon PARP inhibitor-induced were observed to promote cell death in this context.[204] Possibly, targeting resolvase activities could be used to promote mitotic catastrophe, and through this mechanism, may potentiate replication perturbing drugs, including PARP inhibitors.[246]

Immune-checkpoint inhibitors

Defects in genome maintenance components lead to mitotic aberrancies, including chromosome mis-segregation and chromosome bridges in anaphase cells.[204,247] Such unresolved mitotic DNA damage frequently results in micronuclei,[248,249] which are small DNA-containing structures surrounded by one lipid bilayer and are not part of the main nucleus in a cell.[248] Micronuclei that originate from mitotic DNA damage typically contain acentric chromosome fragments (in contrast to mis-segregation events which lead to micronuclei containing entire chromosomes). Chromatin in micronuclei is unable to support faithful DNA replication and DNA repair, leading to additional DNA damage, including local chromosome shattering (i.e. 'chromothripsis').[217,250] Importantly, the nuclear lamina of micronuclei is not properly organized and frequently ruptures, leading to the release of micronucleus DNA into the cytoplasm.[251]

Cells have evolved an innate immune response against viral or bacterial DNA, which utilizes cytosolic DNA sensors, including the cyclic GMP-AMP synthase (cGAS).[252] Like microbial DNA, also 'self' DNA that ends up in the cytosol following a failed mitosis is recognized by cGAS.[253,254] Activated cGAS subsequently catalyzes the production of cyclic GAMP, which activates STING-dependent inflammatory signaling, including the production of type-1 interferons (IFNs).[253-255] Interestingly, the cytoplasmic DNA induced by irradiation promoted immune checkpoint inhibition-mediated tumor clearance by anti-CTLA4 antibodies, in a STING-dependent fashion (Figure 4).[253] Whether these observations also apply to tumors with cytoplasmic DNA due to the consequences of replication stress needs to be established. Similarly, investigation

whether immune-checkpoint inhibition results in increased tumor clearance in situations of tumor-intrinsic or therapy-induced replication stress is warranted.

Biomarkers to identify tumors with replication stress

Analyzing replication stress is challenging due to the broad definition of this process and the lack of direct cellular markers.[53] In experimental settings, replication stress is commonly analyzed by assessing the activation status of the DNA damage response kinase ATR, and the phosphorylation status of its substrates, including CHK1 (at Ser-317 and Ser-345), RPA32 (also called RPA2, at Ser-33), and H2AX (at Ser-139, in phosphorylated form referred to γ-H2AX).[123,124,129,256] These markers become apparent in response to the generation of ssDNA, which indeed reflect replication stress.[125,130] Yet, other events, including resection of DSBs also result in ssDNA generation, and lead to positivity of the same post-translational events.[257] Also, ATR is activated during normal replication,[258] and positivity of these markers should be considered in reference to normal proliferating cells. Along the same line, γ-H2AX also reflects DSBs, which are not related to replication forks. Here, a distinction is made between foci-specific γ-H2AX and pan-nuclear γ-H2AX, with the latter reflecting replication stress.[259] Alternatively, γ-H2AX staining could be combined with markers of S phase, including PCNA foci staining or EdU incorporation to more selectively identify cells with replication stress.

A more direct measurement of replication stress is the assessment of nucleotide incorporation at replication forks. The DNA fiber analysis or DNA combing offers the possibility to study the fork progression dynamics. Specifically, incorporated thymidine analogues (*i.e.* IdU, CldU, BrdU and EdU) can be visualized and quantified. When IdU and CldU are successively incorporated into ongoing replication forks, the length of replication 'fibers' can be visualized in stretched or 'combed' DNA.[260] Depending on the treatment and order of labeling, DNA fiber analysis can determine the velocity of replication, rates of origin firing, symmetry of fork progression within a replication bubble, and ability of cells to protect nascent DNA at stalled forks.[90,165261-263] Although this assay accurately measures replication and replication stress, it is not suited for analysis of fixed tissues, including paraffin-embedded patient samples. Especially in this context, we still rely on proxy measurements. Reliable markers of replication stress that can be used to characterize tumor material are especially welcome to allow patient selection in the context of treatment with agents that target replication stress.

6. CONCLUDING REMARKS

Replication stress in tumor cells leads to genomic instability and through this mechanism can promote mutagenesis and promote tumor development. Yet, replication stress adversely affects the viability of tumor cells. As a result, tumor cells may become addicted to pathways that enable tumor cells to deal with high levels of replication stress (so-called non-oncogene addition).[264] Multiple agents have been developed to target such mechanisms, including cell cycle checkpoint kinases. Increasing knowledge on the biological mechanisms that enable (cancer) cells to resolve or survive replication-associated DNA lesions will further aid in developing new approaches to better treat patients with such cancers.

REFERENCES

1. Network TCGAR, Koboldt DC, Fulton RS, et al. Comprehensive molecular portraits of human breast tumours. Nature. 2012;

2. Bell D, Berchuck A, Birrer M, et al. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;

3. Ciriello G, Miller ML, Aksoy BA, et al. Emerging landscape of oncogenic signatures across human cancers. Nat Genet. 2013;

4. Gaillard H, García-Muse T, Aguilera A. Replication stress and cancer. Nature Reviews Cancer. 2015.

5. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. Science. 2008.

6. Leonard AC, Mechali M. DNA replication origins. Cold Spring Harb Perspect Med. 2013;

7. Linskens MH, Huberman J A. Organization of replication of ribosomal DNA in Saccharomyces cerevisiae. Mol Cell Biol. 1988;

8. Mesner LD, Valsakumar V, Karnani N, et al. Bubble-chip analysis of human origin distributions demonstrates on a genomic scale significant clustering into zones and significant association with transcription. Genome Res. 2011;

9. Bell SP, Dutta A. DNA Replication in Eukaryotic Cells. Annu Rev Biochem. 2002;

10. Méchali M. Eukaryotic DNA replication origins: Many choices for appropriate answers. Nature Reviews Molecular Cell Biology. 2010.

11. Moyer JD, Barbacci EG, Iwata KK, et al. Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. Cancer Res. 1997;

12. Pacek M, Walter JC. A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. EMBO J. 2004;

13. Kunkel TA, Burgers PM. Dividing the workload at a eukaryotic replication fork. Trends in Cell Biology. 2008.

14. Pursell ZF, Isoz I, Lundström EB, et al. Yeast DNA polymerase epsilon participates in leading-strand DNA replication. Science. 2007;

15. King RW, Deshaies RJ, Peters JM, et al. How proteolysis drives the cell cycle. Science. 1996.

16. Nurse P. Universal control mechanism regulating onset of M-phase. Nature. 1990.

17. Malumbres M, Harlow E, Hunt T, et al. Cyclin-dependent kinases: a family portrait. Nat Cell Biol. 2009;

18. Ishida S, Huang E, Zuzan H, et al. Role for E2F in Control of Both DNA Replication and Mitotic Functions as Revealed from DNA Microarray Analysis. Mol Cell Biol. 2001;

19. Ren B, Cam H, Takahashi Y, et al. E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. Genes Dev. 2002;

20. Nasmyth K. Control of the yeast cell cycle by the Cdc28 protein kinase. Curr Opin Cell Biol. 1993;

21. Jackson AL, Pahl PM, Harrison K, et al. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol. 1993;

22. Kitada K, Johnson AL, Johnston LH, et al. A multicopy suppressor gene of the Saccharomyces cerevisiae G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. Mol Cell. 1993;

23. Yoon HJ, Loo S, Campbell JL. Regulation of Saccharomyces cerevisiae CDC7 function during the cell cycle. Mol Biol Cell. 1993;

24. Deegan TD, Yeeles JT, Diffley JF. Phosphopeptide binding by Sld3 links Dbf4-dependent kinase to MCM replicative helicase activation. EMBO J. 2016;

25. Kumagai A, Shevchenko A, Shevchenko A, et al. Treslin Collaborates with TopBP1 in Triggering the Initiation of DNA Replication. Cell. 2010;

26. Kumagai A, Shevchenko A, Shevchenko A, et al. Direct regulation of Treslin by cyclin-dependent kinase is essential for the onset of DNA replication. J Cell Biol. 2011;

27. Liu E, Li X, Yan F, et al. Cyclin-dependent Kinases Phosphorylate Human Cdt1 and Induce Its Degradation. J Biol Chem. 2004;

28. Sugimoto N, Tatsumi Y, Tsurumi T, et al. Cdt1 Phosphorylation by Cyclin A-dependent Kinases Negatively Regulates Its Function without Affecting Geminin Binding. J Biol Chem. 2004;

29. Arias EE, Walter JC. PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. Nat Cell Biol. 2006;

30. Senga T, Sivaprasad U, Zhu W, et al. PCNA is a cofactor for CDT1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. J Biol Chem. 2006;

31. Klotz-Noack K, McIntosh D, Schurch N, et al. Re-replication induced by geminin depletion occurs from G2 and is enhanced by checkpoint activation. J Cell Sci. 2012;

32. McGarry TJ, Kirschner MW. Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell. 1998;

33. Melixetian M, Ballabeni A, Masiero L, , et al. Loss of Geminin induces rereplication in the presence of functional p53. J Cell Biol. 2004;

34. Tada S, Li A, Maiorano D, et al. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol. 2001;

35. Wohlschlegel JA, Dwyer BT, Dhar SK, et al. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science. 2000;

36. Zhu W, Chen Y, Dutta A. Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. Mol Cell Biol. 2004;

37. Perkins G, Drury LS, Diffley JFX. Separate SCFCDC4recognition elements target Cdc6 for proteolysis in S phase and mitosis. EMBO J. 2001;

38. Walter D, Hoffmann S, Komseli ES, et al. SCFCyclin F-dependent degradation of CDC6 suppresses DNA re-replication. Nat Commun. 2016;

39. Méndez J, Zou-Yang XH, Kim SY, et al. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell. 2002;

40. Diffley JFX. Regulation of early events in chromosome replication. Current Biology. 2004.

41. Aparicio OM. Location, location, location: It's all in the timing for replication origins. Genes and Development. 2013.

42. Hayano M, Kanoh Y, Matsumoto S, et al. Rif1 is a global regulator of timing of replication origin firing in fission yeast. Genes Dev. 2012;

43. Knott SR V, Peace JM, Ostrow AZ, et al. Forkhead transcription factors establish origin timing and long-range clustering in S. cerevisiae. Cell. 2012;

44. Tazumi A, Fukuura M, Nakato R, et al. Telomere-binding protein Taz1 controls global replication timing through its localization near late replication origins in fission yeast. Genes Dev. 2012;

45. Cornacchia D, Dileep V, Quivy JP, et al. Mouse Rif1 is a key regulator of the replication-timing programme in mammalian cells. EMBO J. 2012;

46. Foti R, Gnan S, Cornacchia D, et al. Nuclear Architecture Organized by Rif1 Underpins the Replication-Timing Program. Mol Cell. 2016;

47. Yamazaki S, Ishii A, Kanoh Y, et al. Rif1 regulates the replication timing domains on the human genome. EMBO J. 2012;

48. Cayrou C, Coulombe P, Vigneron A, et al. Genome-scale analysis of metazoan replication origins reveals their organization in specific but flexible sites defined by conserved features. Genome Res. 2011;

49. Devbhandari S, Jiang J, Kumar C, et al. Chromatin Constrains the Initiation and Elongation of DNA Replication. Mol Cell. 2017;

50. Kurat CF, Yeeles JTP, Patel H, et al. Chromatin Controls DNA Replication Origin Selection, Lagging-Strand Synthesis, and Replication Fork Rates. Mol Cell. 2017;

51. Lubelsky Y, Sasaki T, Kuipers MA, et al. Pre-replication complex proteins assemble at regions of low nucleosome occupancy within the Chinese hamster dihydrofolate reductase initiation zone. Nucleic Acids Res. 2011;

52. MacAlpine HK, Gordân R, Powell SK, et al. Drosophila ORC localizes to open chromatin and marks sites of cohesin complex loading. Genome Res. 2010;

53. Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nature Cell Biology. 2014.

54. Sheiness D, Michael Bishop J. DNA and RNA from Uninfected Vertebrate Cells Contain Nucleotide Sequences Related to the Putative Transforming Gene of Avian Myelocytomatosis Virus. JOURNAL OF VIROLOGY. 1979.

55. Blackwood EM, Eisenman RN. Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science. 1991;

56. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. Nature Reviews Molecular Cell Biology. 2005.

57. Eilers M, Schirm S, Bishop JM. The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J. 1991;

58. Freytag SO, Geddes TJ. Reciprocal regulation of adipogenesis by Myc and C/EBPα. Science. 1992;

59. Adams JM, Harris AW, Pinkert CA, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature. 1985;

60. Dalla-Favera R, Bregni M, Erikson J, et al. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci. 1982;

61. *Erikson J, Nishikura K, ar-Rushdi A, et al. Translocation of an immunoglobulin kappa locus to a region 3' of an unrearranged c-myc oncogene enhances c-myc transcription. Proc Natl Acad Sci U S A. 1983;*

62. *Gelmann EP, Psallidopoulos MC, Papas TS, et al. Identification of reciprocal translocation sites within the c-myc oncogene and immunoglobulin μ locus in a Burkitt lymphoma. Nature. 1983;*

63. *Nesbit CE, Tersak JM, Prochownik E V. MYC oncogenes and human neoplastic disease. Oncogene. 1999.*

64. *Bouchard C, Dittrich O, Kiermaier A, et al. Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. Genes Dev. 2001;*

65. *Fernandez PC, Frank SR, Wang L, et al. Genomic targets of the human c-Myc protein. Genes Dev. 2003;*

66. *Hermeking H, Rago C, Schuhmacher M, et al. Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A. 2000;*

67. *Staller P, Peukert K, Kiermaier A, et al. Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol. 2001;*

68. *Wu S, Cetinkaya C, Munoz-Alonso MJ, et al. Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. Oncogene. 2003;*

69. *Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. Cell. 2004;*

70. *Miliani de Marval PL, Macias E, Rounbehler R, et al. Lack of Cyclin-Dependent Kinase 4 Inhibits c-myc Tumorigenic Activities in Epithelial Tissues. Mol Cell Biol. 2004;*

71. *Campaner S, Doni M, Hydbring P, et al. Cdk2 suppresses cellular senescence induced by the c-myc oncogene. Nat Cell Biol. 2010;*

72. *Seoane J, Le H Van, Massagué J. Myc suppression of the p21Cip1 Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature. 2002;*

73. *Evan GI, Wyllie AH, Gilbert CS, et al. Induction of apoptosis in fibroblasts by c-myc protein. Cell. 1992;*

74. *Shi Y, Glynn JM, Guilbert LJ, et alRole for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science. 1992;*

75. *Srinivasan SV., Dominguez-Sola D, Wang LC, et al. Cdc45 Is a Critical Effector of Myc-Dependent DNA Replication Stress. Cell Rep. 2013;*

76. *Valovka T, Schönfeld M, Raffeiner P, et al. Transcriptional control of DNA replication licensing by Myc. Sci Rep. 2013;*

77. *Dominguez-Sola D, Ying CY, Grandori C, et al. Non-transcriptional control of DNA replication by c-Myc. Nature. 2007;*

78. *Vafa O, Wade M, Kern S, et al. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: A mechanism for oncogene-induced genetic instability. Mol Cell. 2002;*

79. *Karlsson A, Deb-Basu D, Cherry A, et al. Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. Proc Natl Acad Sci. 2003;*

80. *Felsher DW, Bishop JM. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci. 1999;*

81. *Reimann M, Loddenkemper C, Rudolph C, et al. The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. Blood. 2007;*

82. *Dulić V, Lees E, Reed SI. Association of human cyclin e with a periodic G1-S phase protein kinase. Science. 1992;*

83. *Koff A, Cross F, Fisher A, et al. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell. 1991;*

84. *Koff A, Giordano A, Desai D, et al. Formation and activation of a cyclin E-cdk2 complex during the G 1 phase of the human cell cycle. Science. 1992;*

85. *Harbour JW, Luo RX, Dei Santi A, et al. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell. 1999;*

86. *Nevins JR. The Rb/E2F pathway and cancer. Hum Mol Genet. 2001;*

87. *Jansen-Dürr P, Meichle A, Steiner P, et al. Differential modulation of cyclin gene expression by MYC. Proc Natl Acad Sci U S A. 1993;*

88. *Ekholm-Reed S, Méndez J, Tedesco D, et al. Deregulation of cyclin E in human cells interferes with prereplication complex assembly. J Cell Biol. 2004;*

89. *Bester AC, Roniger M, Oren YS, et al. Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell. 2011;*

90. *Jones RM, Mortusewicz O, Afzal I, et al. Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress. Oncogene. 2013;*

91. *Bartkova J, Hořejší Z, Koed K, et al.*

DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005;

92. *Ohtsubo M, Roberts JM. Cyclin-dependent regulation of G1in mammalian fibroblasts. Science. 1993;*

93. *Resnitzky D, Gossen M, Bujard H, et al. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol Cell Biol. 1994;*

94. *Burrell RA, McClelland SE, Endesfelder D, et al. Replication stress links structural and numerical cancer chromosomal instability. Nature. 2013;*

95. *Spruck CH, Won KA, Reed SI. Deregulated cyclin E induces chromosome instability. Nature. 1999;*

96. *Bos JL. Ras Oncogenes in Human Cancer: A Review. Cancer Res. 1989;*

97. *Filmus J, Robles AI, Shi W, Wong MJ, et al. Induction of cyclin D1 overexpression by activated ras. Oncogene. 1994;*

98. *Peeper DS, Upton TM, Ladha MH, et al. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature. 1997;*

99. *Di Micco R, Fumagalli M, Cicalese A, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature. 2006;*

100. *Liu J, Chao J, Jiang M, et al. Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol Cell bilogy. 1995;*

101. *Maya-Mendoza A, Ostrakova J, Kosar M, et al. Myc and Ras oncogenes engage different energy metabolism programs and evoke distinct patterns of oxidative and DNA replication stress. Mol Oncol. 2015;*

102. *Kotsantis P, Silva LM, Irmscher S, et al. Increased global transcription activity as a mechanism of replication stress in cancer. Nat Commun. 2016;*

103. *Irani K, Xia Y, Zweier JL, et al. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 1997;*

104. *Weyemi U, Lagente-Chevallier O, Boufraqech M, et al. ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. Oncogene. 2012;*

105. *Wilhelm T, Ragu S, Magdalou I, et al. Slow Replication Fork Velocity of Homologous Recombination-Defective Cells Results from Endogenous Oxidative Stress. PLoS Genet. 2016;*

106. *Abulaiti A, Fikaris AJ, Tsygankova OM, et al. Ras induces chromosome instability and abrogation of the DNA damage response. Cancer Res. 2006;*

107. *Macheret M, Halazonetis TD. Intragenic origins due to short G1 phases underlie oncogene-induced DNA replication stress. Nature. 2018;*

108. *Aguilera A, García-Muse T. R Loops: From Transcription Byproducts to Threats to Genome Stability. Molecular Cell. 2012.*

109. *Thomas M, White RL, Davis RW. Hybridization of RNA to double-stranded DNA: formation of R-loops. PNAS. 1976;*

110. *White RL, Hogness DS. R loop mapping of the 18S and 28S sequences in the long and short repeating units of drosophila melanogaster rDNA. Cell. 1977;*

111. *Yu K, Chedin F, Hsieh CL, et al. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat Immunol. 2003;*

112. *El Hage A, French SL, Beyer AL, et al. Loss of Topoisomerase I leads to R-loop-mediated transcriptional blocks during ribosomal RNA synthesis. Genes Dev. 2010;*

113. *Roy D, Zhang Z, Lu Z, et al. Competition between the RNA Transcript and the Nontemplate DNA Strand during R-Loop Formation In Vitro: a Nick Can Serve as a Strong R-Loop Initiation Site. Mol Cell Biol. 2010;*

114. *Baldacci G, Cherif-Zahar B, Bernardi G. The initiation of DNA replication in the mitochondrial genome of yeast. Embo J. 1984;*

115. *Pohjoismäki JLO, Holmes JB, Wood SR, et al. Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid. J Mol Biol. 2010;*

116. *Xu B, Clayton DA. RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers. EMBO J. 1996;*

117. *Westover KD. Structural Basis of Transcription: Separation of RNA from DNA by RNA Polymerase II. Science. 2004;*

118. *Gan W, Guan Z, Liu J, et al. R-loop-mediated genomic instability is caused by impairment of replication fork progression. Genes Dev. 2011;*

119. *Hamperl S, Cimprich KA. Conflict Resolution in the Genome: How Transcription and Replication Make It Work. Cell. 2016.*

120. *Sollier J, Stork CT, García-Rubio ML, et al. Transcription-Coupled Nucleotide Excision Repair Factors Promote R-Loop-Induced Genome Instability. Mol Cell. 2014;*

121. *Helmrich A, Ballarino M, Tora L. Collisions between Replication and*

Transcription Complexes Cause Common Fragile Site Instability at the Longest Human Genes. Mol Cell. 2011;

122. *Stork CT, Bocek M, Crossley MP, et al. Co-transcriptional R-loops are the main cause of estrogen-induced DNA damage. Elife. 2016;*

123. *Wold MS. REPLICATION PROTEIN A:A Heterotrimeric, Single-Stranded DNA-Binding Protein Required for Eukaryotic DNA Metabolism. Annu Rev Biochem. 1997;*

124. *Lupardus PJ, Byun T, Yee MC, et al. A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. Genes Dev. 2002;*

125. *You Z, Kong L, Newport J. The role of single-stranded DNA and polymerase α in establishing the ATR, Hus1 DNA replication checkpoint. J Biol Chem. 2002;*

126. *Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003;*

127. *Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. Nature Reviews Molecular Cell Biology. 2008.*

128. *Cortez D, Guntuku S, Qin J, et al. ATR and ATRIP: Partners in checkpoint signaling. Science. 2001;*

129. *Cimprich KA, Cortez D. ATR: An essential regulator of genome integrity. Nature Reviews Molecular Cell Biology. 2008.*

130. *Zou L, Liu D, Elledge SJ. Replication protein A-mediated recruitment and activation of Rad17 complexes. PNAS, 2003;*

131. *Bass TE, Luzwick JW, Kavanaugh G, et al. ETAA1 acts at stalled replication forks to maintain genome integrity. Nat Cell Biol. 2016;*

132. *Haahr P, Hoffmann S, Tollenaere MAX, et al. Activation of the ATR kinase by the RPA-binding protein ETAA1. Nat Cell Biol. 2016;*

133. *Matsuoka S, Ballif BA, Smogorzewska A, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science. 2007;*

134. *Kumagai A, Dunphy WG. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. Mol Cell. 2000;*

135. *O'Connell MJ, Raleigh JM, Verkade HM, et al. Chk1 is a wee1 kinase in the G2DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J. 1997;*

136. *Boutros R, Dozier C, Ducommun B. The when and wheres of CDC25 phosphatases. Current Opinion in Cell Biology. 2006.*

137. *Furnari B, Rhind N, Russell P. Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. Science. 1997;*

138. *Karlsson-Rosenthal C, Millar JBA. Cdc25: mechanisms of checkpoint inhibition and recovery. Trends in Cell Biology. 2006.*

139. *Sanchez Y, Wong C, Thoma RS, et al. Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25. Science. 1997;*

140. *Siliciano JD, Canman CE, Taya Y, et al. DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev. 1997;*

141. *Tibbetts RS, Brumbaugh KM, Williams JM, et al. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev. 1999;*

142. *Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. p53-Deficient Cells Rely on ATM- and ATR-Mediated Checkpoint Signaling through the p38MAPK/MK2 Pathway for Survival after DNA Damage. Cancer Cell. 2007;*

143. *Reinhardt HC, Hasskamp P, Schmedding I, et al. DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. Mol Cell. 2010;*

144. *Kopper F, Bierwirth C, Schon M, et al. Damage-induced DNA replication stalling relies on MAPK-activated protein kinase 2 activity. Proc Natl Acad Sci. 2013;*

145. *Shechter D, Costanzo V, Gautier J. ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol. 2004;*

146. *Katsuno Y, Suzuki A, Sugimura K, et al. Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc Natl Acad Sci. 2009;*

147. *Marheineke K, Hyrien O. Control of replication origin density and firing time in Xenopus egg extracts. Role of a caffeine-sensitive, ATR-dependent checkpoint. J Biol Chem. 2004;*

148. *Maya-Mendoza A, Petermann E, Gillespie DAF, et al. Chk1 regulates the density of active replication origins during the vertebrate S phase. EMBO J. 2007;*

149. *Costanzo V, Shechter D, Lupardus PJ, et al. An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. Mol Cell. 2003;*

150. *Karnani N, Dutta A. The effect of the intra-S-phase checkpoint on origins*

of replication in human cells. Genes Dev. 2011;

151. *Ge XQ, Blow JJ. Chk1 inhibits replication factory activation but allows dormant origin firing in existing factories. J Cell Biol. 2010;*

152. *Chen YH, Jones MJK, Yin Y, et al. ATR-Mediated Phosphorylation of FANCI Regulates Dormant Origin Firing in Response to Replication Stress. Mol Cell. 2015;*

153. *Yekezare M, Gomez-Gonzalez B, Diffley JFX. Controlling DNA replication origins in response to DNA damage - inhibit globally, activate locally. J Cell Sci. 2013;*

154. *Lopes M, Cotta-Ramusino C, Pellicioli A, et al. The DNA replication checkpoint response stabilizes stalled replication forks. Nature. 2001;*

155. *Tercero JA, Diffley JFX. Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature. 2001;*

156. *Couch FB, Bansbach CE, Driscoll R, et al. ATR phosphorylates SMARCAL1 to prevent replication fork collapse. Genes Dev. 2013;*

157. *Lossaint G, Larroque M, Ribeyre C, et al. FANCD2 Binds MCM Proteins and Controls Replisome Function upon Activation of S Phase Checkpoint Signaling. Mol Cell. 2013;*

158. *Forment J V., Blasius M, Guerini I, et al Structure-specific DNA endonuclease mus81/eme1 generates DNA damage caused by chk1 inactivation. PLoS One. 2011;*

159. *Murfuni I, Basile G, Subramanyam S, et al. Survival of the Replication Checkpoint Deficient Cells Requires MUS81-RAD52 Function. PLoS Genet. 2013;*

160. *Técher H, Koundrioukoff S, Carignon S, et al. Signaling from Mus81-Eme2-Dependent DNA Damage Elicited by Chk1 Deficiency Modulates Replication Fork Speed and Origin Usage. Cell Rep. 2016;*

161. *Hu J, Sun L, Shen F, et al. The intra-S phase checkpoint targets Dna2 to prevent stalled replication forks from reversing. Cell. 2012;*

162. *Sogo JM, Lopes M, Foiani M. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science. 2002;*

163. *Neelsen KJ, Lopes M. Replication fork reversal in eukaryotes: From dead end to dynamic response. Nature Reviews Molecular Cell Biology. 2015.*

164. *Berti M, Chaudhuri AR, Thangavel S, et al. Human RECQ1 promotes restart of replication forks reversed by DNA topoisomerase I inhibition. Nat Struct Mol Biol. 2013;*

165. *Schlacher K, Christ N, Siaud N, et al. Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell;*

166. *Schlacher K, Wu H, Jasin M. A Distinct Replication Fork Protection Pathway Connects Fanconi Anemia Tumor Suppressors to RAD51-BRCA1/2. Cancer Cell. 2012;*

167. *Leuzzi G, Marabitti V, Pichierri P, et al. WRNIP1 protects stalled forks from degradation and promotes fork restart after replication stress. EMBO J. 2016;*

168. *Zadorozhny K, Sannino V, Beláň O, et al. Fanconi-Anemia-Associated Mutations Destabilize RAD51 Filaments and Impair Replication Fork Protection. Cell Rep. 2017;*

169. *Mijic S, Zellweger R, Chappidi N, et al. Replication fork reversal triggers fork degradation in BRCA2-defective cells. Nat Commun. 2017;*

170. *Ding X, Chaudhuri AR, Callen E, et al. Synthetic viability by BRCA2 and PARP1/ARTD1 deficiencies. Nat Commun. 2016;*

171. *Chaudhuri AR, Callen E, Ding X, et al. Replication fork stability confers chemoresistance in BRCA-deficient cells. Nature. 2016;*

172. *Rondinelli B, Gogola E, Yücel H, et al. EZH2 promotes degradation of stalled replication forks by recruiting MUS81 through histone H3 trimethylation. Nat Cell Biol. 2017;*

173. *Xu S, Wu X, Wu L, et al. Abro1 maintains genome stability and limits replication stress by protecting replication fork stability. Genes Dev. 2017;*

174. *Thangavel S, Berti M, Levikova M, et al. DNA2 drives processing and restart of reversed replication forks in human cells. J Cell Biol. 2015;*

175. *Feng W, Jasin M. BRCA2 suppresses replication stress-induced mitotic and G1 abnormalities through homologous recombination. Nat Commun. 2017;*

176. *Liang F, Han M, Romanienko PJ, Jasin M. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. PNAS. 1998;*

177. *Johnson RD. Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. EMBO J. 2000;*

178. *Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. Nature. 2014;*

179. *Cejka P. DNA end resection: Nucleases team up with the right partners to initiate homologous recombination. Journal of Biological Chemistry. 2015.*

180. *Kowalczykowski SC. An overview of the molecular mechanismsof recombinational DNA repair. Cold Spring Harbor Perspectives in Biology. 2015.*

181. *West SC, Blanco MG, Chan YW, et al. Resolution of recombination intermediates: Mechanisms and regulation. Cold Spring Harbor Symposia on Quantitative Biology. 2016.*

182. *Yang J, Bachrati CZ, Ou J, et al. Human topoisomerase IIIα is a single-stranded DNA decatenase that is stimulated by BLM and RMI1. J Biol Chem. 2010;*

183. *Wechsler T, Newman S, West SC. Aberrant chromosome morphology in human cells defective for Holliday junction resolution. Nature. 2011;*

184. *D'Andrea AD. Susceptibility Pathways in Fanconi's Anemia and Breast Cancer. N Engl J Med. 2010;*

185. *Sawyer SL, Tian L, Kähkönen M, et al. Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype. Cancer Discov. 2015;*

186. *Howlett NG, Taniguchi T, Durkin SG, et al. The Fanconi anemia pathway is required for the DNA replication stress respone and for the regulation of common fragile site stability. Hum Mol Genet. 2005;*

187. *Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet. 2007;*

188. *Kim H, D'Andrea AD. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes and Development. 2012.*

189. *Cervenka J, Arthur D, Yasis C. Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. Pediatrics. 1981;*

190. *Taniguchi T, Tischkowitz M, Ameziane N, et al. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med. 2003;*

191. *McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. Cancer Res. 2006;*

192. *Chan KL, Palmai-Pallag T, Ying S, et al. Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. Nat Cell Biol. 2009;*

193. *Yang W, Gao Y. Translesion and Repair DNA Polymerases: Diverse Structure and Mechanism. Annu Rev Biochem. 2018;*

194. *Moldovan GL, Pfander B, Jentsch S. PCNA, the Maestro of the Replication Fork. Cell. 2007.*

195. *Hoege C, Pfander B, Moldovan GL, et al. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature. 2002;*

196. *Watanabe K, Tateishi S, Kawasuji M, et al. Rad18 guides polη to replication stalling sites through physical interaction and PCNA monoubiquitination. EMBO J. 2004;*

197. *Kannouche PL, Wing J, Lehmann AR. Interaction of human DNA polymerase η with monoubiquitinated PCNA: A possible mechanism for the polymerase switch in response to DNA damage. Mol Cell. 2004;*

198. *Ghosal G, Chen J. DNA damage tolerance: a double-edged sword guarding the genome. Transl Cancer Res. 2013;*

199. *Mateos-Gomez PA, Gong F, Nair N, et al. Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. Nature. 2015;*

200. *Kawamura K, Bahar R, Seimiya M, et al. DNA polymerase θ is preferentially expressed in lymphoid tissues and upregulated in human cancers. Int J Cancer. 2004;*

201. *Ceccaldi R, Liu JC, Amunugama R, et al. Homologous-recombination-deficient tumours are dependent on Polθ -mediated repair. Nature. 2015;*

202. *Goullet de Rugy T, Bashkurov M, Datti A, et al. Excess Polθ functions in response to replicative stress in homologous recombination-proficient cancer cells. Biol Open. 2016;*

203. *Minocherhomji S, Ying S, Bjerregaard VA, et al. Replication stress activates DNA repair synthesis in mitosis. Nature. 2015;*

204. *Schoonen PM, Talens F, Stok C, et al. Progression through mitosis promotes PARP inhibitor-induced cytotoxicity in homologous recombination-deficient cancer cells. Nat Commun. 2017;*

205. *Wyatt HDM, Laister RC, Martin SR, et al. The SMX DNA Repair Tri-nuclease. Mol Cell. 2017;*

206. *Chan YW, West SC. Spatial control of the GEN1 Holliday junction resolvase ensures genome stability. Nat Commun. 2014;*

207. *Naim V, Wilhelm T, Debatisse M, Rosselli F. ERCC1 and MUS81-EME1 promote sister chromatid separation by processing late replication intermediates at common fragile sites during mitosis. Nat Cell Biol. 2013;*

208. *Ying S, Minocherhomji S, Chan KL, et al. MUS81 promotes common fragile site expression. Nat Cell Biol. 2013;*

209. *Duda H, Arter M, Gloggnitzer J, et al. A Mechanism for Controlled Breakage of Under-replicated Chromosomes during Mitosis. Dev Cell. 2016;*

210. *Chan YW, Fugger K, West SC. Unresolved recombination intermediates lead to ultra-fine anaphase bridges, chromosome breaks and aberrations. Nat Cell Biol. 2018;*

211. *Mankouri HW, Huttner D, Hickson ID. How unfinished business from S-phase affects mitosis and beyond. EMBO Journal. 2013.*

212. *Tiwari A, Addis Jones O, Chan KL. 53BP1 can limit sister-chromatid rupture and rearrangements driven by a distinct ultrafine DNA bridging-breakage process. Nat Commun. 2018;*

213. *Baumann C, Körner R, Hofmann K, et al. PICH, a Centromere-Associated SNF2 Family ATPase, Is Regulated by Plk1 and Required for the Spindle Checkpoint. Cell. 2007;*

214. *Biebricher A, Hirano S, Enzlin JH, et al. PICH: A DNA Translocase Specially Adapted for Processing Anaphase Bridge DNA. Mol Cell. 2013;*

215. *Ke Y, Huh JW, Warrington R, et al. PICH and BLM limit histone association with anaphase centromeric DNA threads and promote their resolution. EMBO J. 2011;*

216. *Hengeveld RCC, de Boer HR, Schoonen PM, et al. Rif1 Is Required for Resolution of Ultrafine DNA Bridges in Anaphase to Ensure Genomic Stability. Dev Cell. 2015;*

217. *Zhang CZ, Spektor A, Cornils H, et al. Chromothripsis from DNA damage in micronuclei. Nature. 2015;*

218. *Gorgoulis VG, Vassiliou LVF, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005;*

219. *Courtois-Cox S, Jones SL, Cichowski K. Many roads lead to oncogene-induced senescence. Oncogene. 2008.*

220. *Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. 1983;*

221. *Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). Cell. 1997;*

222. *Sherr CJ. Cancer cell cycles. Science. 1996.*

223. *Enoch T, Carr AM, Nurse P. Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes Dev. 1992;*

224. *Toledo LI, Altmeyer M, Rask MB, et al. ATR prohibits replication catastrophe by preventing global exhaustion of RPA. Cell. 2014.*

225. *Toledo L, Neelsen KJ, Lukas J. Replication Catastrophe: When a Checkpoint Fails because of Exhaustion. Molecular Cell. 2017.*

226. *Belanger F, Angers J-P, Fortier E, et al. Mutations in Replicative Stress Response Pathways Are Associated with S Phase-specific Defects in Nucleotide Excision Repair. J Biol Chem. 2016;*

227. *Luo M, He H, Kelley MR, et al. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. Antioxid Redox Signal. 2010;*

228. *Ichikawa J, Tsuchimoto D, Oka S, et al. Oxidation of mitochondrial deoxynucleotide pools by exposure to sodium nitroprusside induces cell death. DNA Repair. 2008;*

229. *Oka S, Ohno M, Tsuchimoto D, et al. Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. EMBO J. 2008;*

230. *Sakumi K, Furuichi M, Tsuzuki T, et al. Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. J Biol Chem. 1993;*

231. *Gad H, Koolmeister T, Jemth AS, et al. MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool. Nature. 2014;*

232. *Löbrich M, Jeggo PA. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer. 2007;*

233. *Kawabe T. G2 checkpoint abrogators as anticancer drugs. Mol Cancer Ther. 2004;*

234. *Lecona E, Fernández-Capetillo O. Replication stress and cancer: It takes two to tango. Experimental Cell Research. 2014.*

235. *Matheson CJ, Backos DS, Reigan P. Targeting WEE1 Kinase in Cancer. Trends in Pharmacological Sciences. 2016.*

236. *Wieringa HW, van der Zee AGJ, de Vries EGE, vet al. Breaking the DNA damage response to improve cervical cancer treatment. Cancer Treatment Reviews. 2016.*

237. *Schoppy DW, Ragland RL, Gilad O, Set al. Oncogenic stress sensitizes murine cancers to hypomorphic suppression of ATR. J Clin Invest. 2012;*

238. *Murga M, Campaner S, Lopez-Contreras AJ, et al. Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. Nat Struct Mol Biol. 2011;*

239. *Ruiz S, Mayor-Ruiz C, Lafarga V, et al. A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors. Mol Cell. 2016;*

240. *Chen X, Low KH, Alexander A, et al. Cyclin E overexpression sensitizes triple negative breast cancer to Wee1 kinase Inhibition. Clin Cancer Res. 2018;*

241. *Dietlein F, Thelen L, Jokic M, et al. A functional cancer genomics screen identifies a druggable synthetic lethal interaction between MSH3 and PRKDC. Cancer Discov. 2014;*

242. *Kabeche L, Nguyen HD, Buisson R, et al. A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation. Science. 2018;*

243. *Krajewska M, Fehrmann RSN, Schoonen PM, et al. ATR inhibition preferentially targets homologous recombination-deficient tumor cells. Oncogene. 2015;*

244. *Abdel-Fatah TMA, Middleton FK, Arora A, et al. Untangling the ATR-CHEK1 network for prognostication, prediction and therapeutic target validation in breast cancer. Mol Oncol. 2015;*

245. *Lai X, Broderick R, Bergoglio V, et al. Corrigendum: MUS81 nuclease activity is essential for replication stress tolerance and chromosome segregation in BRCA2-deficient cells. Nat Commun. 2017;*

246. *Schoonen PM, van Vugt MATM. Never tear us a-PARP: Dealing with DNA lesions during mitosis. Mol Cell Oncol. 2018;*

247. *Laulier C, Cheng A, Stark JM. The relative efficiency of homology-directed repair has distinct effects on proper anaphase chromosome separation. Nucleic Acids Res. 2011;*

248. *Lewis CW, Golsteyn RM. Cancer cells that survive checkpoint adaptation contain micronuclei that harbor damaged DNA. Cell Cycle. 2016;*

249. *Tkacz-Stachowska K, Lund-Andersen C, Velissarou A, et al. The amount of DNA damage needed to activate the radiation-induced G2 checkpoint varies between single cells. Radiother Oncol. 2011;*

250. *Crasta K, Ganem NJ, Dagher R, et al. DNA breaks and chromosome pulverization from errors in mitosis. Nature. 2012.*

251. *Hatch EM, Fischer AH, Deerinck TJ, et al. Catastrophic Nuclear Envelope Collapse in Cancer Cell Micronuclei. Cell. 2013;*

252. *Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. Nature Immunology. 2016.*

253. *Harding SM, Benci JL, Irianto J, et al. Mitotic progression following DNA damage enables pattern recognition within micronuclei. Nature. 2017;*

254. *MacKenzie KJ, Carroll P, Martin CA, et al. CGAS surveillance of micronuclei links genome instability to innate immunity. Nature. 2017;*

255. *Bakhoum SF, Ngo B, Laughney AM, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. Nature. 2018;*

256. *Rogakou EP, Pilch DR, Orr AH, et al. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. 1998;*

257. *Jazayeri A, Falck J, Lukas C, et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol. 2006;*

258. *Saldivar JC, Hamperl S, Bocek MJ, et al. An intrinsic S/G2checkpoint enforced by ATR. Science. 2018;*

259. *Murga M, Bunting S, Montãa MF, et al. A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat Genet. 2009;*

260. *Bensimon A, Simon A, Chiffaudel A, et al Alignment and sensitive detection of DNA by a moving interface. Science. 1994;*

261. *Bryant HE, Petermann E, Schultz N, et al. PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. EMBO J. 2009;*

262. *Maya-Mendoza A, Moudry P, Merchut-Maya JM, et al. High speed of fork progression induces DNA replication stress and genomic instability. Nature. 2018;*

263. *Petermann E, Orta ML, Issaeva N, et al. Hydroxyurea-Stalled Replication Forks Become Progressively Inactivated and Require Two Different RAD51-Mediated Pathways for Restart and Repair. Mol Cell. 2010;*

264. *Luo J, Solimini NL, Elledge SJ. Principles of Cancer Therapy: Oncogene and Non-oncogene Addiction. Cell. 2009.*